

Effect of *Rotula aquatica* Lour. on Experimental Kidney Stones

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ABSTRACT

Urinary kidney stone formation is multifaceted complex process involving accumulation and aggregation of oxalates and phosphates of calcium, magnesium, ammonium and uric acid. Traditional systems of healing like Ayurveda and Unani have played a praiseworthy role from time immemorial in treatment of kidney stones. One such a plant is *Rotula aquatica* Lour. belonging to Borage family. In Ayurveda, it is described as '*Paashanabhedha*' meaning 'stone breaking'. It is highly endorsed with diuretic, laxative properties and used in treatment of kidney stones. The present study was taken up to evaluate the antiurolithiatic potential of the roots by a methodical approach, using simple *in-vitro* model. Calcium oxalate and phosphate mainly compose the kidney stones. Hence the study involved the preparation of Calcium oxalate and phosphate and its dissolution in presence of *Rotula aquatica* Lour. The successive solvent extracts were screened for *in-vitro* antiurolithiatic activity using semi-permeable membrane of eggs. The aqueous extract had high dissolving potential of 29.182% for Ca-Ox and 65.445 % for CaPO₄. A trend of dose dependent dissolution was identified with increasing the concentration of aqueous extract. 100% dissolution was found at 38 and 20mg of aqueous extract for Ca-Ox and CaPO₄ respectively. Phyto-sterols, phenolic compounds like flavonoids and tannins, reducing sugars and amino acids were present in the aqueous extract. On HPLC analysis of aqueous extract five major peaks with R_t of 2.433, 5.308, 7.365, 10.436, 10.857 min were significant at 205nm. The TLC profile was established in Chloroform: ethanol (9:1) solvent system with seven bands at 254 nm and two bands at 366nm. Ten bands were derivatised after spraying with vanillin-sulphuric acid. The present study reveals the promising evidence of *Rotula aquatica* Lour. used as '*Paashanabhedha*' used in traditional system.

Keywords: *Rotula aquatica* Lour., *in-vitro* antiurolithiatic activity, Ca-Ox, CaPO₄, aqueous extract, dissolution, TLC profile, HPLC

INTRODUCTION

Urolithiasis or nephrolithiasis is the common term used for lithiasis in the kidneys and urinary tract. Stones < 3 mm/ 0.12") cause obstruction of the ureter leading to pain in flank, lower abdomen and painful micturation. 75 % of urinary calculi is composed of pure calcium oxalate (50 %) or calcium phosphate (5 %) or a mixture of both (45 %) ¹. Other combinations include that of magnesium, ammonium salts and of uric acid. Epidemiological studies reveals that, nephrolithiasis is more common in men of about 12% than in women of about 6%, prevalent between the ages of 20 to 40 in both sexes ². The worldwide incidence of urolithiasis is quite high in spite of advances in the field of medicine. Increased rate of hypertension, obesity are linked to nephrolithiasis and contribute to increase in stone formation ³.

The pathogenesis of calcium stone formation is a multi-step process which includes-nucleation, crystal growth, crystal aggregation and crystal retention ⁴. Increased urinary retention of stone forming constituent elements like Calcium, phosphorus, uric acid, oxalate and physico-chemical change like pH influence urolithiasis. The goals of treatment include reduction of super saturation of urine by rehydration, reduce the reoccurrence rate by avoiding dietary calcium supplements, treating cellular

injury using analgesics, antioxidants and infections with antibiotics ⁵. When the size of stone is > 3mm surgical procedures are the only final option. However its worth noted that high reoccurrence rate is the self-limitation of both non-surgical and surgical methods ⁶. Drugs of natural origin (including herbal medicine) have always been accepted as the choice of treatment by over 80% of the world's population ⁷. In most of the traditional systems of medicine remedies are taken from plants ⁸. In Ayurveda, the term *Paashanabhedha* is used for a group of plants that dissolve kidney stones and uric acid crystals ^{9,10}. *Pasha* means -stone, *bhedha* means -break. Many plants like *Bergenia stracheyi* Hk., *Bergenia ciliata* (Haw.)Sternb., *Coleus forskohlii* Briq., *Kalanchoe pinnata* (Lam.) pers., *Aerva lanata* Juss are marketed as '*Paashanabhedha*'. One among them is *Rotula aquatica* Lour. ¹¹. *Rotula aquatica* Lour, belonging to family Boraginaceae is a species of rare rheophytic woody aromatic flowering medicinal shrub native to India and part of the lotic ecosystem of streams ¹². The whole plant and roots possess bitter, astringent, sudorific, antilithic properties. Traditionally roots are used in hemorrhoids, renal and vesicle calculi, diabetes and in venereal diseases. The diuretic action of root is attributed to presence of allantoin ¹³. Scientific studies on the plant include for its

Table 1: Nature and % yield of extracts

Extract	% yield*	Nature of extract	
		Color	Consistency
Pet ether	1.0802 ± 0.04	Yellowish	Sticky
Chloroform	0.722 ± 0.039	Dark brown	Crystalline powder
n-butanol	1.226 ± 0.023	Dark brown	Hygroscopic powder
Ethyl acetate	0.14 ± 0.044	Greenish brown	Sticky
Ethanol	0.991 ± 0.012	Dark brown	Amorphous powder
Methanol	0.2461 ± 0.0387	Dark brown	Amorphous powder
Aqueous	4.840 ± 0.0299	Dark brown	Crystalline powder

* mean ±SD

Table 2: Optimization of solvent system aqueous extract

Solvent system	Detection by UV @254nm		Detection by Vanillin sulphuric acid	
	No. of bands	of Rf	No. of bands	Rf value
Me-OH: water: A. acid (6:4:0.1)	-	-	2	0.27, 0.95
EA: Me-OH: water (7:2:1)	-	-	2	0.95, 0.98
Toulene: EA (8: 2)	1	0.98	2	0.67, 0.98
EA: Me-OH: A. acid (2:2:0.5)	-	-	2	0.86, 0.94
n-Bu-OH : GAA : water(4:1:5)	-	-	2	0.66, 0.74
Chloroform: methanol (8:2)	-	-	2	0.90, 0.95
Chloroform: ethanol (9:1)	3	0.42, 0.53, 0.72	6	0.27, 0.37, 0.45, 0.58, 0.72, 0.87, 0.95

- 'Indicates no separation

antimitotic¹⁴, antibacterial¹⁵, urolithiatic^{16,17}, anthelmintic¹⁸, antidiarrheal¹⁹, analgesic, anti-inflammatory, antipyretic²⁰ and antioxidant²¹ properties. Hence, the present study was carried out to find the efficacy of *Rotula aquatica* Lour, claiming the traditional use Paashanabheda and its bioactivity guided fractionation for antiurolithiatic activity.

MATERIALS AND METHODS

The dried roots of *Rotula aquatica* Lour, were collected from Malappuram District, Kerala and authenticated by Dr. Santhan P, Plant taxonomist, Natural remedies Pvt. Ltd, Bangalore. A voucher specimen RA/R/04 was also deposited at the herbarium of Department of Pharmacognosy, Government College of Pharmacy, Bengaluru. The roots were thoroughly washed 2-3 times in running water then with distilled water and air dried on a blotter under shade. The air dried roots were coarsely ground using a Remi Laboratory Mixer and sieved to #24 and stored in airtight containers. The extracts were prepared by successive solvent extraction in a soxhlet assembly with solvents in the increasing order of polarity viz. pet-ether 60-80^o C, chloroform, n-butanol, ethyl-acetate, ethanol, and methanol. The aqueous extract was obtained by distillation. The extracts were concentrated in a Rotavapor (Buchi) at 30^o C. The color, consistency of the extracts were noted and stored at 4± 1.0^o C.

Preliminary phytochemical screening

By following standard procedures qualitative chemical tests were conducted on the successive solvent extracts to identify the presence of various phytoconstituents like alkaloids, flavonoids, phenols saponins, steroids, terpenoids, proteins and amino acids²².

Experimental design

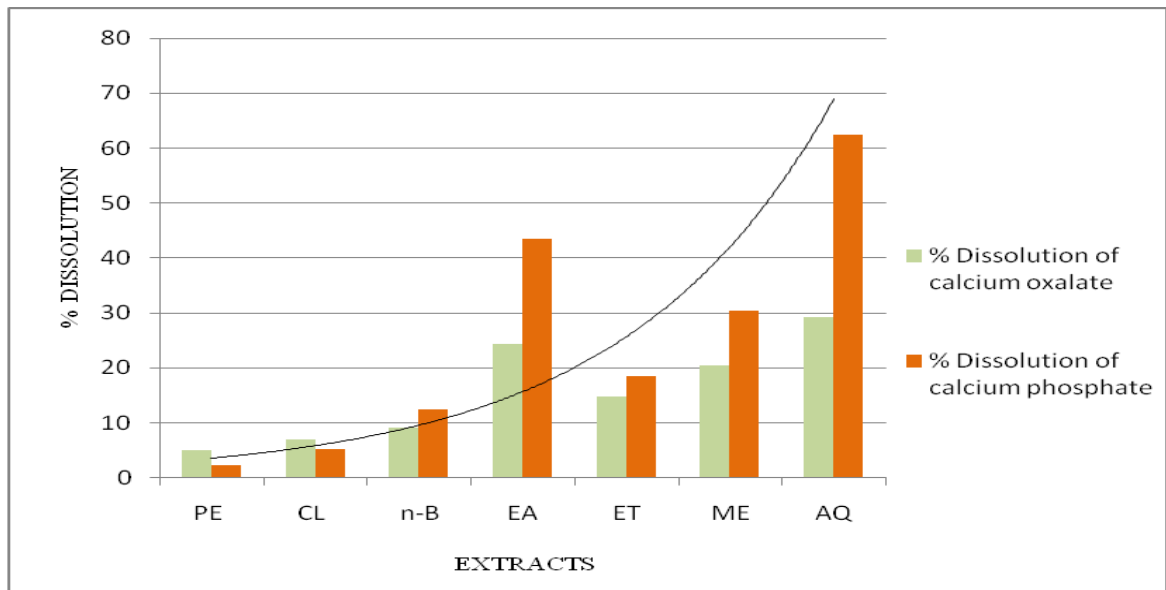
The effect of various extracts on the dissolution of calcium oxalate (Ca-Ox) and calcium phosphate (Ca-PO₄) using the semi permeable membrane (SPM) of from farm eggs was determined by titrimetric method²³ and UV spectrophotometric method²⁴ respectively.

In-Vitro Antiurolithiatic Activity

The SPM was prepared by decalcification of eggs and removal of its inner liquid contents. The prepared SPM was placed in 2% ammonia solution until used. A homogenous precipitate of crystals was prepared by mixing equimolar solution of acidic solution of CaCl₂.2H₂O, Na-Ox and CaCl₂.2H₂O, disodium hydrogen phosphate for Ca-Ox and Ca-PO₄ respectively. The precipitated stones were freed from traces of acid using ammonia. Finally, the washed stones were dried at 60± 2^o C for 4 hours. 1mg of Ca-Ox/ Ca-PO₄ was placed in SPM along with 10mg of the extract. SPM was carefully suspended in 100ml of 0.1 M Tris buffer. The test group contained Ca-Ox/ Ca-PO₄ along with extract while the control contained only Ca-Ox/ Ca-PO₄. The conical flasks were placed in incubator at 37^o C for 4hrs. For estimation of Ca-Ox, contents of the SPM from each group were transferred in to test tubes. 2ml of 1N sulphuric acid was added and allowed to react for 2-3minutes and titrated against 0.9494N KMNO₄ till light pink end point. The amount of Calcium in control and test groups was calculated using the factor 1ml of 0.9494N KMNO₄ ≡ 0.1898mg of Ca²⁺. The % Dissolution of each of the extracts was calculated using the formula.

$$\% \text{ Dissolution} = \frac{\text{Weight of Ca}^{2+} \text{ reduced}}{\text{Weight of Ca}^{2+} \text{ estimated}} \times 100$$

$$[\text{weight of ca}^{2+} \text{ reduced} = (\text{wt of ca}^{2+} \text{ estimated})_{\text{control}} - (\text{wt of ca}^{2+} \text{ estimated})_{\text{test}}]$$



PE-pet ether, CL- chloroform, n-B- n- butanol, EA- ethylacetate, Et- ethanol, ME- methanol, AQ- aqueous extracts

Figure 1: % Dissolution of Ca-Ox and Ca-Po4 by extracts

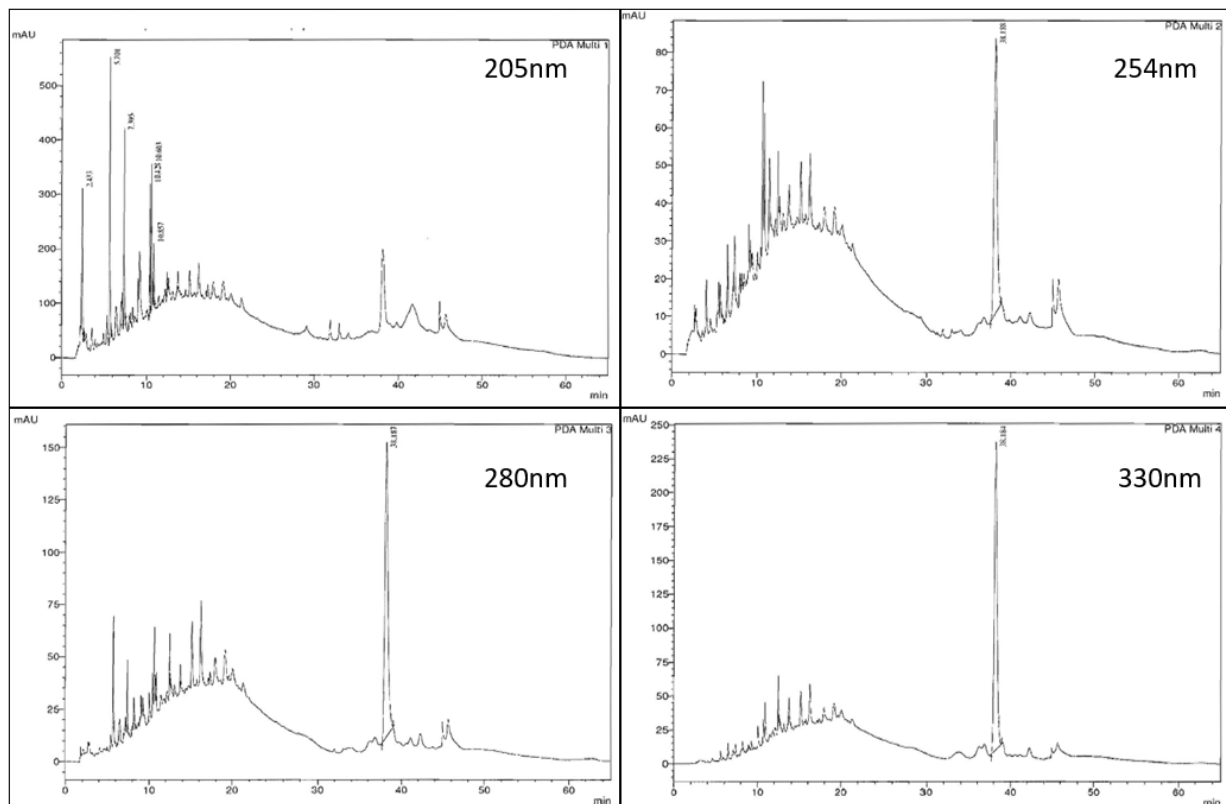
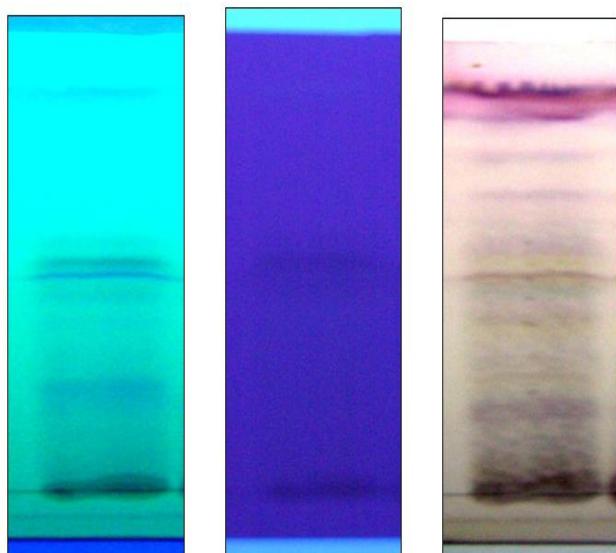


Figure 2: HPLC analysis of Aqueous extract

For estimation of Ca-PO₄, the contents of the SPM were carefully transferred in to 10ml volumetric flasks. Standard solutions of Ca-PO₄ were prepared with 100-800 µg/ml. to each flask 2ml of 1N sulphuric acid; 2.8ml of molybdic H₂SO₄ reagent and 1ml if reducing solution of p-phenylene diamine were added to test and standard and made up to volume with distilled water. The color produced was measured at 600-750nm. The undissolved Ca-PO₄ was determined by extrapolation of graph. All

experiments were performed in triplicate with a control group. The average % dissolution of each of the extracts was calculated as mean ± standard deviation. From the above studies, it was found that the aqueous extract had significant activity compared to other extracts. Thus the aqueous extract was subjected to HPLC and TLC analysis.

HPLC evaluation of the aqueous extract



TLC of aqueous extract at 254nm, 366nm, after spraying vanillin-sulphuric acid

Figure 3: TLC profiling of aqueous extract

HPLC evaluation of the aqueous extract was done by dissolving 40mg of extract in HPLC grade water (Millipore) in Shimadzu HPLC system LC 2010A, detected with UV PDA detector and analysed with LC solution software. The solvents were filtered through 0.45 μ membrane filter and degassed in sonicator for 3 minutes. RP C₁₈, 5 μ m (250 X 4.6 mm) column was eluted with A: orthophosphoric acid and potassium dihydrogen orthophosphate in HPLC grade water and B: Acetonitrile.

TLC profiling:

TLC profile of aqueous extract was developed by the method of optimization using different solvent systems to show maximum separation. 2mg of aqueous fraction was dissolved in water: methanol. The sample was applied as thin bands on silica gel 60 F₂₅₄ (0.25 μ) precoated plates (Merck). The plates were developed in different solvent systems and air dried. Visualization was done in day light, 254 and 366nm. Later the plates were sprayed with vanillin-sulphuric acid and heated in hot air oven (maintained at 105 $^{\circ}$ C) for 15minutes. The R_f values were calculated and recorded²⁵.

RESULTS

In the present study, the roots of *Rotula aquatica* Lour. were selected to study the antiurolithiatic potential by a methodical approach of using simple *in-vitro* model based on its traditional use. The nature and yield of successive solvent extracts were recorded and are given in Table 1. The preliminary screening for antiurolithiatic activity by using SPM of eggs was carried out on all the extracts accordingly. The percentage dissolution of Ca-Ox by the successive solvent extracts were found to be 5.167%, 7.092%, 9.092%, 24.399%, 14.783%, 20.559%, 29.182% for pet ether, chloroform, n-butanol, ethyl acetate, ethanol, methanol and aqueous extract respectively. Similarly the percentage dissolution of CaPO₄ by the successive solvent extracts were found to

be 2.262, 5.338, 12.395, 43.459, 18.623, 30.478, 65.445 % for pet ether, chloroform, n-butanol, ethyl acetate, ethanol, methanol and aqueous extract respectively. From the above studies, it was found that the aqueous extract had significant activity compared to other extracts (Fig.1). Further the preliminary phytochemical screening of aqueous extract indicated the presence of phyto-sterols, phenolic compounds like flavonoids and tannins, reducing sugars and amino acids. Previous studies on *Rotula aquatica* Lour. by Sasikala et al.²⁶, by nucleation and demineralization assay also indicated that the active constituents responsible for its antiurolithiatic potential is present in the aqueous extract. As a step ahead towards identifying the principal constituents HPLC analysis (Fig. 2) and TLC profiles (Table 2) are established in the present investigation. On analysis of HPLC chromatogram it was found that the majority of peaks were laid within the retention time of 30 minutes indicating its polar nature. Five major peaks with R_t of 2.433, 5.308, 7.36, 10.43, 10.85 min were significant at 205nm with % area of 11.042, 80.43, 34.64, 26.54, 4.69 respectively. At 254, 280 and 305 nm a distinct peak with maximum % area of 94.37 at R_t of 33.18 min was recorded. The TLC studies in Chloroform: ethanol (9:1) showed the presence of seven bands in UV 254 nm with R_f of 0.27, 0.42, 0.47, 0.53, 0.66, 0.72, 0.91, two bands with R_f 0.53 and 0.66 in UV 366nm. After spraying with vanillin-sulphuric acid, ten bands with R_f of 0.27, 0.37, 0.42, 0.47, 0.53, 0.58, 0.72, 0.87, 0.91, 0.95 were obtained.

DISCUSSION

Urolithiasis is the third most common disorder of the urinary tract, the others being frequently occurring urinary tract infections and benign prostatic hyperplasia/prostate diseases⁷⁻¹⁰. Since the main intention of treatment is enhancing excretion of stone forming constituent elements and altering the physico-chemical environment which influence formation many drugs are lined up in the market. However with current therapy the risks of reoccurrence are too high. Thus there is retrieval of interest towards safer plant drugs. The presented study on the *in-vitro* antiurolithiatic activity on *Rotula aquatica* Lour. shows that there is greater dissolution of Ca-Ox and CaPO₄ by the aqueous extract than the other extracts. The trend of dose dependent dissolution was identified with increasing the concentration. The diuretic property attributed to the plant can be inferred from the presented *in-vitro* antiurolithiatic dissolution study. From the HPLC and TLC studies it is evident that the aqueous extract contains many constituents which are also likely to contribute to its property of "*Paashanabhedha- the stone breaker*". Further the constituents responsible for the antiurolithiatic potential are under investigation.

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Abbreviations

Ca-Ox- Calcium Oxalate, CaPO₄- Calcum Phosphate, TLC-Thin Layer Chromatogram, R_f- Retention Factor, HPLC- High Performance Liquid Chromatography, R_t- Retention Time, µ-Micron, µm-Micrometer

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